RESEARCH ARTICLE

A duplex polymerase chain reaction-restriction fragment length polymorphism for rapid screening of methylenetetrahydrofolate reductase gene variants: Genotyping in acute leukemia

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Background: Methylenetetrahydrofolate reductase (*MTHFR*; NM_005957.4) is the key enzyme for folate metabolism which plays in DNA biosynthesis and the epigenetic process of DNA methylation. *MTHFR* gene polymorphisms, the c. 677C>T and c. 1298A>C have been implicated as risk factors for several types of cancers as the acute leukemia.

Aim: We have optimized a duplex polymerase chain reaction-restriction fragment length polymorphism assay (PCR-RFLP) for the simultaneous detection of both variants in acute leukemia patients, from Tunisia.

Methods: Genomic DNA was extracted from EDTA-anticoagulant blood samples from a total of 50 patients suffering from acute leukemia (AL). After DNA extraction, the polymerase chain reaction using specific primers, designed using Primer 3 Software. Restriction Fragment Length Polymorphism (RFLP) was performed in two separate tubes followed by agarose gel electrophoresis.

Conclusion: This new method has proved to be a rapid, simple, and reliable method that should facilitate high throughput genotyping of *MTHFR* polymorphisms in acute leukemia.

KEYWORDS

methylenetetrahydrofolate reductase, polymerase chain reaction, polymorphisms, restriction fragment length polymorphism

1 | INTRODUCTION

Methylenetetrahydrofolate reductase (*MTHFR*; NM_005957.4) is the key enzyme for folate metabolism which plays in DNA biosynthesis and the epigenetic process of DNA methylation.

MTHFR gene polymorphisms, the c. 677C>T and c. 1298A>C, have been implicated as risk factors for several complex disease¹ with emphasis on essential hypertension,² Parkinson's disease,³ Alzheimer disease⁴ pregnancy complications,⁵ and various cancers such acute leukemia.⁶⁻⁸

We have optimized a duplex polymerase chain reaction-restriction fragment length polymorphism assay (PCR-RFLP) for the simultaneous detection of both variants in acute leukemia patients, from Tunisia.

2 | MATERIALS AND METHODS

Our study included 50 patients suffering from acute leukemia (AL).

Genomic DNA was extracted from EDTA-anticoagulant blood samples according to salting protocol.⁹

PCR amplification was performed using specific primers, designed using Primer 3 Software, for both interested SNPs of *MTHFR* gene (Figures 1 and 2). These primers are not affected by dimers formation.

Each 50 µL reaction contained approximately 60 ng genomic DNA, 10 pmoles/mL of each primer, and a master mix (PCR Super Mix; cat 10572-014 Invitrogen; Life Technologies Europe BV, Bleiswijk.).

UCSC In-Silico PCR

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>chr1:11856293-11856402 110bp
TGAAGGAGAAGGTGTCTGCGGGA
GCAAGTGATGCCCATGTCGGTG
TGAAGGAGAAGGTGTCTGCGGGAgccgatttcatcatcacgcagctt
tctttgaggctgacacattctccgctttggaaggcatgCACCGACATGGG
CATCACTTGC
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FIGURE 1 Sequences of primers for the exon 4 of *MTHFR* (110 bp)

UCSC In-Silico PCR

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>chr1:11854313-11854498 186bp
GGGGAGGAGCTGACCAAGTGA
AGGGCACAGGATGGGGAAGT
GGGGAGGAGCTGACCAAGTGAagaagtgtctttgaagtcttcttctt
acctctcgggagaaccaaaccggaatgggtcacaaagtgtgatgctggagt
gggacctgttcatccctgccctggcctgacccagctgcaggccaggctgc
ggggctgtgACTTCCCCATCCTGTGCCCT
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FIGURE 2 Sequences of primers for the exon 7 of *MTHFR* (186 bp)

FIGURE 3 Duplex polymerase chain reaction and restriction fragment length polymorphism with *HinfI* for *MTHFR* c.677C>T. AL: allelic leader (50bp); 2; 3 and 6: duplex PCR of *MTHFR* (110 bp for exon 4 and 186 bp for exon 7); 1; 4 and 5: RFLP of *MTHFR* with *HinfI*; 1 and 4: CT-Heterozygote and 5: CC wild Type

PCR parameters consisted of an initial denaturation step at 95°C for 8 minutes, followed by 35 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute, and finally 8 minutes at 72°C.

Methods	PCR-RFLP	PCR-sequencing	HRM
Processing time	3H	>24H	1.5 H
Cost and suitability	Low, adapted for large samples	High, adapted to small samples	High, adapted to small samples
Reproducibility	Good	Good	Good
Accuracy	Accurate	Accurate	Gold standard

PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; HRM, high resolution melting.

To identify *MTHFR* c.677C>T and c.1298A>C, *HinfI* and *MbolI* restriction enzyme were used, respectively, in separate tubes.

A 3% agarose gel electrophoresis was performed at 80 V during 20 minutes. The amplicons from PCR and PCR-RFLP were visualized by ethidium bromide under an UV transilluminator.

To validate our newly developed multiplex PCR, DNA samples were randomly selected for sequencing.

3 | RESULTS

In the present study, we established a duplex PCR-RFLP assay to detect both variants of *MTHFR*. Several factors, such as primer concentration and PCR cycling condition, were optimized.

Simultaneous amplification of both SNPs of *MTHFR* (NM_005957.4:c.677C>T and c.1298C>A) was performed and the sizes of the PCR products generated from our primers for exon 4 and 7 of *MTHFR* were 110 bp and 186 bp respectively.

A presence of *HinfI* recognition site (5'-G▼ANCC-3') with the T allele in *MTHFR* (NM_005957.4:c.677C>T) produces digested products, resulted in 110, 87 and 23 bp. However, a presence of *MbolI* restriction site (5'-G A A G A(N)8▼-3') with the A allele in *MTHFR* (NM_005957.4:c.1298C>A) results in 156 bp and 30 bp fragments.

Among our study, eight patients were heterozygous for c.677C>T of *MTHFR* (677CT) with an overall frequency of 16% (Figure 3).

4 | DISCUSSION

We have successfully developed a new and simple method for the simultaneous detection of the *MTHFR* c.677C>T and c.1298A>C and genotyping of 50 patients with AL.

For diagnostic analysis of a large number of patients, the fast and economic assay is highly desirable. Thus, a number of molecular approaches targeting genomic DNA for identification of both polymorphisms have also been developed (Table 1). These include different types of conventional PCR, real-time PCR, and DNA sequencing.¹⁰⁻¹² All of these techniques are fast and elegant, but they require expensive equipment. Because of their cost and complexity, they might be not used routinely in some medical environments.

Our assay presents several main advantages. Firstly, a one step amplification for both SNPs, secondly, no special equipment is used, and thirdly the results are stable and reproducible.

TABLE 1 Comparison of genotyping methods

Both variants were screened in the AL patients. Interestingly, we have eight heterozygous 677 CT of *MTHFR* cases with an overall frequency of 16%. This result highlights the possible impact of C677T polymorphism on the risk of AL, however, never *MTHFR* c.1298A>C (as 1298 AC or 1298 CC) was found (0%).

In fact, it has been proved that the *MTHFR* 677TT genotype conferred a significant higher risk of leukemia.¹³ In addition, *MTHFR* 677TT genotype may be associated with elevated risk of adverse effects during Methotrexate treatment of AL patients.¹⁴

5 | CONCLUSION

This duplex PCR-RFLP provided an early and accurate diagnosis of the *MTHFR* c.677C>T and c.1298A>C; hence it may be used as an alternative method to the conventional gold technique for the rapid genotyping of *MTHFR* polymorphisms in acute leukemia.

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